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INDUCTION, MULTIPLICATION, AND ACCLIMATIZATION OF RED BETEL PLANT (PIPER CROCATUM RUIZ AND PAV.) BY IN VITRO ORGANOGENESIS

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Graphical abstract

Abstract

Red betel is an herbal plant with anticancer activities. Propagation of red betel by conventional vegetative method has low multiplication factors. The purpose of this research was to increase the propagation rate of red betel by utilizing in vitro culture organogenesis method which was consisted of stages as follows: shoots initiation, shoots multiplication, root induction, and acclimatization. Shoots induction from axillary bud was achieved on MS 3 mgL-1 BAP media. Shoots were multiplicated on MS (0, 0.5, 1, 1.5 mgL-1) BAP, (0.01, 0.03, 0.1, 0.3 mgL-1) TDZ, and (0.01, 0.03, 0.05, 0 mgL-1) TDZ combined with 0.5 mgL-1 NAA. Roots were induced on MS (0, 0.5, 1, 1, 5 mgL-1) NAA. The maximum number of shoots (4.4 ± 0.9) was achieved on MS 1 mgL-1 BAP. The maximum number of roots (22.8 ± 9.7) was achieved on MS 0.5 mgL-1 NAA. The highest survival rates of red betel plants during acclimatization and post-acclimatization periods were 88% and 100%, respectively. Red betel had been successfully multiplicated by in vitro direct organogenesis.

Keywords: Piper crocatum ruiz and pav, in vitro organogenesis, induction, multiplication, acclimatization

1.0 INTRODUCTION

Red betel (Piper crocatum Ruiz and Pav.) is a medicinal herb which has a high potency for curing various diseases, because it has anticancer [1], antihyperglycemic, antidiabetic [2], antiinflammation [3], and antibacterial activities [4]. Red betel is also effective in curing whiteness [5] and acts as natural antioxidants [6]. Red betel contains a broad array of phytochemical compounds such as alkaloids, flavonoids, tannins, and saponins [3, 7].

Red betel has been cultivated by vegetative methods in its natural habitat, specifically by cutting and grafting methods [8]. However, propagation of red betel plant through vegetative methods has limitations, such as the short storage period of planting materials, slow rate of plant multiplication, and susceptibility to viral infections [9].

The problems of conventional propagation can be overcomed by tissue culture method. Tissue culture is able to produce high-quality planting materials, uniform seeds, and disease-resistant plants rapidly [10]. Direct organogenesis is one of the propagation methods involving the formation of buds or organs directly without passing through the callus. Propagation can be achieved through the formation of shoots from axillary bud explants by breaking the apical dominance of the apical meristem [11], such as in the case of propagation of Piper nigrum [12].

Murashige Skoog (MS) is the most commonly used basal media in plant tissue culture because it contains macro- and micronutrient to support optimum plant growth. Composition of plant growth regulators in tissue culture media is an instrumental in morphogenesis. Cytokinins is a class of plant growth regulator which is frequently used in propagating various plants in vitro. Cytokinins regulate the formation of axillary buds and lateral roots [11].

The most common types of cytokinins are 6-Benzyl Amino Purine (BAP) and Thiadiazuron (TDZ). BAP is able to induce the growth of axillary bud from shoot tip culture. Multiplication of shoots, buds, and meristems can be optimized by adjusting the concentration of BAP. Low concentration of TDZ is also able to propagate shoots [11]. In vitro herbal plants were successfully induced and multiplicated by using BAP and TDZ. Shoots multiplication of *Talinum triangular* [13], *Gynura pseudochina* (L) DC) [14], and *Gloriosa superba* [15] were achieved on media supplemented with BAP. Meanwhile, *Bacopa monnieri* L was multiplicated on media supplemented with TDZ [16]. Plants of the genus *Piper*, such as black pepper (*Piper nigrum*) and *Piper longum* [17] were also successfully propagated by in vitro culture method [12]. Red betel plantlets were needed to be acclimatized because the success of plant propagation by in vitro method was indicated by the survival rates of plants during acclimatization [10]. Research about in vitro culture of Indonesian red betel plant had never been published before. The specific aim of this research was to obtain the optimal media formulation for in vitro organogenesis of red betel which was consisted of shoots initiation, shoots multiplication, root induction, and acclimatization

2.0 EXPERIMENTAL

2.1 Sterilization of Explants

In vitro red betel plantlets were initiated from a single node axillary bud in Biotechnology Laboratory of Pelita Harapan University. The explants were washed with detergent and rinsed with running water to remove soil particles. The explants were sunk in 0.25 gram of both bactericide and fungicide in 200 ml of water for 30 minutes, 2.25 mgL^{-1} of antibiotic rifampicin for four hours, 2% of Clorox® bleach for 15 minutes, 1.5% of Clorox® bleach for 15 minutes, 1% of Clorox® bleach for 10 minutes, and 0.1% of $HgCl_2$ for 10 minutes. The explants were then rinsed twice with sterile water and planted into in vitro plant tissue culture media in order to induce the growth of explants.

2.2 Induction and Initiation of Shoots

Induction was achieved on MS media supplemented with 3 mgL^{-1} BAP in three months. The parameters recorded in the experiment were the contamination percentages and the mean numbers of shoots produced per explant. The explants were first initiated on MS media supplemented with 3 mg BAP for four months. Explants were then initiated for the second time on MS media supplemented with either 3 mgL^{-1} BAP or 4 mgL^{-1} BAP. The obtained in vitro shoots were used as explants for the next research stages. The parameters recorded in the experiment were the mean number of shoots and leaves produced per explant in two months.

2.3 Multiplication of Shoots

In vitro shoots were multiplicated on MS media supplemented with BAP, TDZ, and TDZ combined with 1-Naphthaleneacetic acid (NAA). Explants were cultured on MS media supplemented with different concentration of BAP (0, 0.5 mgL^{-1}, 1 mgL^{-1}, 1.5 mgL^{-1}), and TDZ (0.01 mgL^{-1}, 0.03 mgL^{-1}, 0.1 mgL^{-1}, 0.3 mgL^{-1}). Explants were also cultured on MS media supplemented with different concentration of TDZ (0.01 mgL^{-1}, 0.03 mgL^{-1}, 0.05 mgL^{-1}, 0 mgL^{-1}) combined with 0.5 mgL^{-1} NAA. The parameters recorded in the experiment were the mean number of shoots and leaves produced and the mean number of shoot lengths in three months.

2.4 Root Induction

Roots were induced on MS media supplemented with (0 mgL^{-1}, 0.5 mgL^{-1}, 1 mgL^{-1}, 1.5 mgL^{-1}) NAA. All of the experiments were replicated 10 times. The parameters recorded in the experiment were the percentage of viable rooted plantlets, time needed for root formation, and the mean number of roots in three months.

2.5 Acclimatization

In vitro propagated plantlets of red betel were acclimatized after three months being cultured in rooting media. The plantlets were washed with running tap water and sunk into the mixture of 0.25 gram bactericide and fungicide in 200 ml of water. The plantlets were then planted into acclimatization media which contained husk and compost (1:1) in plastic bottles. The media were always kept moist in room temperature. The survival rates of plants were recorded for four weeks. Viable plants were then moved into post-acclimatization media composed of a mixture of husk, soil, and compost media (1:1:1) in the green house. The survival rates of plantlets were also recorded for three months.

3.0 RESULTS AND DISCUSSION

3.1 Induction and Initiation of Explants

Multiple shoots were induced from one out of 15 single nodes of axillary buds (6.67%). Multiple shoots were transferred to the first initiation MS media supplemented with 3 mgL^{-1} BAP. The mean number of shoots and leaves were recorded after four months (Table 1). Red betel explants were then initiated on the second initiation media, i.e. MS supplemented with 3 mgL^{-1} BAP and 4 mgL^{-1} BAP. The highest mean numbers of shoots and leaves were obtained on MS supplemented 3 mgL^{-1} BAP, with 10.25 shoots and 30.75 leaves, respectively (Table 1, Figure 1a, b)

Table 1 Mean number of Shoots and Leaves at 12th week

First Initiation (month)[a]	Mean Number of Shoots[c]	Mean of Number of Leaves[c]
1	5.33	10.67
2	6.11	13.56
3	7.44	14.89
4	8	16
Second Initiation (mg^{-1} L)[b]	**Mean Number of shoots[c]**	**Mean Number of leaves[c]**
BAP 3	10.25	30.75
BAP 4	7	22.8

[a] The explants were first initiated on MS BAP 3 mg L^{-1}, [b] Explants were then initiated for the second time on MS BAP 3 mg^{-1} L or BAP 4 mg^{-1} L, [c] the parameters were observed after 12 weeks.

The Formation of shoots and leaves during the induction and initiation phases was influenced by the concentration of cytokinins in the media. BAP is a cytokinin-type plant growth regulator that stimulates the proliferation of axillary buds if it is supplemented in appropriate concentrations [10, 18]. BAP affects the amount of nitrogen and potassium on MS media which are involved in protein synthesis and cell division [19]. The high concentration of BAP in culture media inhibits the differentiation and multiplication of shoots due to the excessive accumulation of cytokinins in the shoots [20].

3.2 Multiplication of Shoots

Multiplication of red betel in vitro plantlets on MS supplemented with BAP obtained the highest mean number of shoots (4.4 ± 0.9), shoot lengths (2.7 ± 1.4 cm), and leaves (20.2 ± 6.1) on media supplemented with 1 mgL^{-1} BAP. Tukey test at 5% significance level showed that the mean number of shoots on MS media supplemented with 1 mgL^{-1} BAP was significantly different from control but not significantly different with MS supplemented with 0.5 mgL^{-1} BAP and 1.5 mgL^{-1} BAP (Table 2; Figure 1c).

Table 2 Mean Number of Shoots, Shoot Lengths, and Leaves on MS BAP Media at 12th week

Concentration of BAP (mg^{-1} L)	Mean Number of Shoots (mean ± SD)*	Mean of Shoot lengths (cm) (mean ± SD)*	Mean Number of Leaves (mean ± SD)*
0	2,6^{a} ± 0,8	1,5^{a} ± 0,17	13,8^{ab} ± 4,9
0.5	3,4^{ab} ± 0,9	1,5^{a} ± 0,3	9,2^{a} ± 4,5
1	4,4^{b} ± 0,9	2,7^{a} ±1,4	20,2^{b} ± 6,1
1.5	3,6^{ab} ± 0,5	1,1^{a} ± 0,3	14^{ab} ± 1,6

* Means followed by same letters are not significantly different at p≤ 0.05 according to Tukey multiple range test

MS basal media supplemented with BAP 1 mgL^{-1} gave the highest mean number of shoots, shoot lengths, and number of leaves. In this study, it appeared that the control medium (MS) was also able to induce the production of shoots. It was probably due to the endogenous cytokinins already existed in red betel plantlets that triggered cell differentiation and bud formation [18]. The high concentration of BAP would otherwise inhibit organogenesis in the genus *Piper* [12, 17].

Multiplication of red betel in vitro plantlets on MS supplemented with TDZ obtained the highest mean number of shoots (2.6 ± 1.3) and leaves (13, 8 ± 4, 9) on media supplemented with 0, 01 mgL^{-1} TDZ. The highest mean of shoot lengths (1.7 ± 0.7 cm) was obtained on media supplemented with 0.3 mgL^{-1} TDZ. Tukey test at 5% level proved that the mean number of shoots on MS (control) was not significantly different from the mean number of shoots on supplemented with mgL^{-1} TDZ, 0.03 mgL^{-1} TDZ, 0.1 mgL^{-1} TDZ, and 0.3 mgL^{-1} TDZ (Table 3; Figure 1d)

Table 3 Mean Number of Shoots, Shoot lengths, and Leaves on TDZ Media at 12th week

Concentration of TDZ (mg^{-1} L)	Mean Number of Shoots (mean ± SD)*	Mean of Shoot lengths (cm) (mean ± SD)*	Mean Number of Leaves (mean ± SD)*
0.01	2.6^{a} ± 0.8	1.5^{a} ± 0.17	13.8^{b} ± 4.9
0.03	1.6^{a} ± 0.9	1.6^{a} ± 1.0	6.10^{a} ± 2.3
0.10	1.8^{a} ± 1.1	1.6^{a} ± 0.8	5.90^{a} ± 3.4
0.30	2.2^{a} ± 2.3	1.7^{b} ± 0.7	5.10^{a} ± 0.3

* Means followed by same letters are not significantly different at p≤ 0.05 according to Tukey multiple range test

TDZ is a kind of diphenyl urea plant growth regulator which can directly triggers plant growth and shoots multiplicaton in a similar physiological way as the N- substituted natural cytokinins. TDZ can also induce the synthesis and accumulation of endogenous cytokinins that trigger bud formation [21]. The highest mean number of shoots of red betel was obtained on media supplemented with low concentration of TDZ, as in Scrophularia takesimensis

[22], Stevia rebaudiana [21] and Nyctanthes arbor [23].

Shoots multiplication of red betel plantlets was achieved on media supplemented with TDZ. The highest mean number of leaves was obtained on media not supplemented with TDZ. It is due to the high concentration of endogenous cytokinins in red betel plant. The high concentration of cytkokinins in the media, such as TDZ, therefore will inhibit the formation of leaves [11] because it accumulates endogenous cytokinins [21]. Plant growth regulators can affect plant's growth even if applied in low concentrations. TDZ concentration and duration of application are important factors in organogenesis [24].

Multiplication of red betel in vitro plantlets on MS supplemented with TDZ and NAA obtained the highest mean number of shoots (3.4 ± 1.7) on media supplemented with 0.03 mgL^{-1} TDZ and 0.5 mgL^{-1} NAA. The lowest mean number of shoots (2.0 ± 0.7) was obtained on MS supplemented with 0.5 mgL^{-1} NAA (Table 4; Figure 1e)

Table 4 Mean Number of Shoots, Shoot Lengths, and Leaves on TDZ Combined with NAA Media at 12th week

Concentration of (mg^{-1} L)	Mean Number of (mean ± SD) *	Mean of Shoot lengths (mean ± SD) *	Mean Number of (mean ± SD)
0.01 + 0.5	2.2^{a} ± 0.8	1.4^{a} ± 0.6	5.6^{a} ± 1.5
0.03 + 0.5	3.4^{a} ± 1.7	1.8^{a} ± 0.3	5.7^{a} ± 1.6
0.05 + 0.5	2.8^{a} ± 0.4	2.3^{a} ± 1.4	4.6^{a} ± 1.9
0.00 + 0.5	2.0^{a} ± 0.7	1.5^{a} ± 0.9	6.1^{a} ± 3.4

* Means followed by same letters are not significantly different at p≤ 0.05 according to Tukey multiple range test

Multiplication of red betel plantlets was performed on media supplemented with TDZ and NAA. Media supplemented with cytokinins and auxin can produce shoots more effectively than media supplemented with TDZ solely [25]. The balancing concentration of cytokinins and auxins is a crucial factor for the growth and development of plants. Therefore, both type of plant growth regulators are needed for plant morphogenesis. *H. cariensis* [26], *A. cariensis* [27] also had the highest number of shoots on media supplemented with TDZ and NAA rather than on media supplemented with TDZ solely.

3.3 Root Induction

Red betel shoots were successfully rooted after four weeks. The percentage of red betel plantlets successfully rooted on MS (control), MS supplemented with 0.5 mgL^{-1} NAA, and MS supplemented with 1.5 mgL^{-1} NAA was 100%. Meanwhile, the percentage of red betel plantlets rooted on MS supplemented with 1 mgL^{-1} NAA was 90%. The highest mean number of roots was obtained on MS supplemented 0.5 mgL^{-1} NAA, with 22.8 ± 9.7 roots (Table 5).

Table 5 Mean Number of Roots and Percentage of Rooted Plantlets on Root Induction Media at 12th week

Concentration of NAA (mg^{-1} L)	Mean Number of Roots (mean ± SD) *	Rooting (%)
0	14.6^{a} ± 7.4	100
0.5	22.8^{a} ± 9.7	100
1	14.1^{a} ± 8.1	90
1.5	22.1^{a} ± 9.8	100

* Means followed by same letters are not significantly different at p≤ 0.05 according to Tukey multiple range test

Root induction of Cinchona succirubra [28] and Celastruss paniculatus [29] had been achieved on media supplemented with NAA. Interestingly, roots of red betel plantlets were able to be induced on media without supplementation NAA. This was probably because red betel already contained endogenous auxin Indole Acetic Acid (IAA) that could be translocated to the roots [18]. IAA is biosynthesized in the apical meristem and then translocated basipetally to induce roots formation. NAA is an auxin-type growth regulator that regulates the formation of roots [18]. In this research, NAA had no significant effect on the formation of roots of red betel plantlets.

3.4 Acclimatization of Plantlets

The in vitro propagated plantlets were acclimatized for four weeks in media containing husk : compost 1 : 1 (Figure 1f), and then transferred to the greenhouse for post-acclimatization in media containing husk : soil : compost 1 : 1 : 1 (Figure 1g). Table 6 shows the viable percentage of red betel plants after four weeks being acclimatized and four weeks being post- acclimatized. The highest survival rates of red betel plants during acclimatization and post- acclimatization were 88% and 100%, respectively (Table 6).

Table 6 Survival Rates of Red Betel Plants during Acclimatization and Post-Acclimatization at 4th week

Root Induction Media (mg^{-1} L NAA)	Acclimatization (%)	Post-Acclimatization (%)
0	88 (8/9)	100 (8/8)
0.5	88 (8/9)	100 (8/8)
1	78 (7/9)	100 (7/7)
1.5	88 (8/9)	100 (8/8)

During acclimatization, red betel plants had a high mortality rate, due to the instable environmental conditions [10]. Besides, the low viability in acclimatization was probably caused by the death of root cells which therefore interfered the acquisition of nutrition and water from planting media. Plants in the

genus *Piper* had various survical rates during acclimatization, such as *Piper longum* (70%) [17], and *Piper auritum* Khun (100%) [30]. Survival rate of a plant in the acclimatization period was also influenced by the number of roots. Auxin which was given in the in vitro root induction media affected the physiological reponse of plant growth and development because auxin interacted with cytokinins which were already synthesized in root meristem. Therefore, there was a balance concentration of auxins and cytokinins which was beneficial for plantlet growth. Red betel plants had a survival rate of 100% during post-acclimatization because they had been adapted to environmental conditions during acclimatization

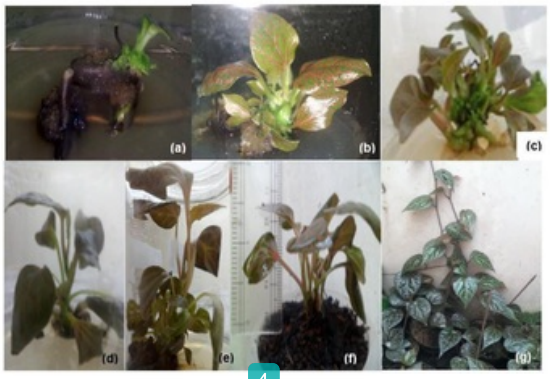

Figure 1 Micropropagation and Acclimatization of Red Betel (Piper crocatum Ruiv and Pav.)

a Shoots Induction of in vitro red b[illegible] on MS supplemented with 3 mgL^{-1} BAP, **b** Shoots Initiation of in vitro red betel o[illegible]S supplemented with 3 mgL^{-1} BAP, **c** Shoots multiplication of in vitro red betel on MS su[illegible]mented with 1 mgL^{-1} BAP, **d** Multiplication of red betel plantlet on MS supplemented w[illegible] nmgL^{-1} TDZ, **e** Shoots multiplication of in vitro red betel on MS supplemented with 0.03 mgL^{-1} TDZ and 0.05 mgL^{-1} NAA, **f** Acclimatization of red betel at first month, **g** post-acclimatization of red betel at third months

4.0 CONCLUSION

Single nodes of red betel explants had been induced and shoots had been initiated on MS supplemented with 3 mgL^{-1} BAP. Shoot multiplication was optimized on MS supplemented with 1 mgL^{-1} BAP. All of the plantlets had been induced to produce roots. The highest mean number of roots had been successfully achieved on MS media supplemented with 0.5 mgL^{-1} NAA. The highest percentage of rooted plantlets was 100%. The highest survival rates of red betel during acclimatization and post acclimatization were 88% and 100%, respectively.

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